Journal Pre-proofs

New guidelines for testing "Deep eutectic solvents" toxicity and their effects on the environment and living beings

Javier Torregrosa-Crespo, Xavier Marset, Gabriela Guillena, Diego J. Ramón, Rosa María Martínez-Espinosa

PII: S0048-9697(19)35375-6
DOI: https://doi.org/10.1016/j.scitotenv.2019.135382
Reference: STOTEN 135382

To appear in: *Science of the Total Environment*

Received Date: 1 August 2019
Revised Date: 8 October 2019
Accepted Date: 3 November 2019

Please cite this article as: J. Torregrosa-Crespo, X. Marset, G. Guillena, D.J. Ramón, R. María Martínez-Espinosa, New guidelines for testing "Deep eutectic solvents" toxicity and their effects on the environment and living beings, *Science of the Total Environment* (2019), doi: https://doi.org/10.1016/j.scitotenv.2019.135382

New guidelines for testing "Deep eutectic solvents" toxicity and their effects on the environment and living beings.

Javier Torregrosa-Crespo[1], Xavier Marset[2], Gabriela Guillena[2], Diego J. Ramón[2], Rosa María Martínez-Espinosa*[1]

[1]Departamento de Agroquímica y Bioquímica. División de Bioquímica y Biología Molecular. Facultad de Ciencias. Universidad de Alicante. Apdo. 99, E-03080-Alicante, Spain.

[2]Instituto de Síntesis Orgánica (ISO) and Departamento de Química Orgánica. Facultad de Ciencias. Universidad de Alicante. Apdo. 99, E-03080-Alicante, Spain.

*Correspondence: rosa.martinez@ua.es; Tel.: +34965903400 ext. 1258; 8841

New guidelines for testing "Deep eutectic solvents" toxicity and their effects on the environment and living beings.

Javier Torregrosa-Crespo[1], Xavier Marset[2], Gabriela Guillena[2], Diego J. Ramón[2], Rosa María Martínez-Espinosa*[1]

[1]Departamento de Agroquímica y Bioquímica. División de Bioquímica y Biología Molecular. Facultad de Ciencias. Universidad de Alicante. Apdo. 99, E-03080-Alicante, Spain.

[2]Instituto de Síntesis Orgánica (ISO) and Departamento de Química Orgánica. Facultad de Ciencias. Universidad de Alicante. Apdo. 99, E-03080-Alicante, Spain.

*Correspondence: rosa.martinez@ua.es; Tel.: +34965903400 ext. 1258; 8841

Highlights

- DESs are promising compounds for organic chemistry and pharmacy.
- Despite their advantages as reaction media, there is controversy about their toxicity.
- Their toxicity was mainly assayed by antibiograms, which is not an accurate approach for DESs.
- New guidelines for DESs toxicity monitoring are presented.
- Protocols to test DES toxicity may lead to errors in assigning their eco-friendly nature.

Abstract

Deep eutectic solvents (DESs) were described at the beginning of this century as an alternative to ionic liquids (ILs) in green chemistry. Despite their obvious sustainable advantages as reaction media, there is still controversy about their potential toxicity. Most of the ecotoxicity assays done up to now involving DESs are based on antibiograms. This is not a good approach due to the high density and viscosity of most DESs already described. Additionally, antibiograms do not allow continuous monitoring of neither cellular growth nor changes on physicochemical parameters like culture acidification due to cellular growth or DESs metabolization. This work starts by displaying advantages and disadvantages of the DESs toxicity assays already reported. Then, using a new DES recently described and *Escherichia coli* as a model microorganism, liquid cultures with continuous monitoring of pH, temperature, shaking and optical density have been used, for the first time, to quantify potential toxicity of the DES as well as the degree of the cellular tolerance (in preadapted and non-preadapted cells). The results obtained show that this new DES is not toxic for *E. coli* at concentrations up to 300 mM and cellular preadaptation was crucial for the cells to grow. At concentrations between 300 mM and 450 mM, cells can tolerate this DES. Above 600 mM, the DES is toxic causing complete inhibition of growth. This toxicity is not only due to the chemical composition of the DES, but also due to the high acidification of the media caused by the DES hydrolysis during cellular growth. The consequences of sterilization procedures on the DES stability are also analysed into detail, finding that sterilization by autoclave promotes DES hydrolysis. From these results, new guidelines are proposed for furthers studies aiming to characterize and quantify DESs toxicity.

Keywords: Deep eutectic solvents, *Escherichia coli*, cellular toxicity, environmental toxicity.

1. Introduction

Deep eutectic solvents (DESs) are solvents generally formed by mixing a variety of quaternary ammonium salts with a Brønsted or Lewis acid compound in certain proportion. They were described for the first time at the beginning of this century as an alternative to ionic liquids (ILs) in green chemistry and catalysis (Abbott et al., 2004; Xu et al., 2017). Since that time around 800 papers have been published about DES (Date of access to PubMed and Scopus: 15 July 2019), involving subjects like: i) synthesis of new DES including their physicochemical characterisation (Marset et al., 2019; Zhang et al., 2012); ii) potential applications of DES as green solvent in biocatalysis (both, in presence or absence of enzymes and/or cells of biotechnological interest) (Gorke et al., 2008; Gutiérrez et al., 2010; Lindberg et al., 2010; Zhao et al., 2011; Lynam et al., 2017; Gotor-Fernández et al., 2019; Pätzold et al., 2019); iii) DES as ingredients for drugs synthesis, solubilization and delivery (Morrison et al., 2009; Pedro et al., 2019; Gutiérrez et al., 2019); iv) DES and/or NADES (Natural Deep Eutectic Solvents) as solvent for extraction of biomolecules like polysaccharides, proteins, alkaloids, phenolic compounds or other natural compounds like chitin, pigments, flavonoids, κ-carrageenan, dyes (Gu et al., 2014; Pena-Pereira et al., 2014; Zeng et al., 2014; García et al., 2016; Zhu et al., 2017; Zhuang et al., 2017; Saravana et al., 2018; Takla et al., 2018; Zdanowicz et al., 2018; Zhang et al., 2018; Huang et al., 2019) or v) DES as media for structural studies of nucleic acids (Mamajanov et al., 2010; Zhao et al., 2013a; Zhao et al., 2013b). More recently, DES-based molecularly imprinted polymers or DES-functionalised nanotubes have been developed and implemented at industrial scale for the removal of contaminants from wastewater or for the screening on antibiotics in biological fluids like milk (AlOmar et al., 2017; Tang et al., 2017; Chen et al., 2018).

Combinations of DES applications with other techniques or approaches such as nanoparticles/nanospheres and electrochemistry have also been reported (Nkuku and LeSuer, 2007; Abbott et al., 2009; Sheng et al., 2012). The main results described up to now reveal DESs as promising solvents not only for green chemical industries (Dai et al., 2013), but also for those related to cosmetics, food, pharmacy, biotechnology and biomedicine (Wagle et al., 2014; Cvjetko Bubalo et al., 2016; Li et al., 2016; Mbous et

al., 2017; Aydin et al., 2018; Jablonský et al., 2018; Procentese et al., 2018; Santos et al., 2018; Yoo et al., 2018).

In order to consider DESs as environmental friendly solvents, their real toxicity as well as their potential biodegradability must be stablished (Radošević et al., 2015). Studies regarding these facts have been performed to a lesser extent; mainly using prokaryotic microbes (Juneidi et al., 2016) and in some cases eukaryotic organisms (Radošević et al., 2016; Dai et al., 2014). Nevertheless, there is still controversy about their potential toxicity (Hayyan et al., 2013a). Whilst several reports support the theory that DESs are non-toxic, eco-friendly, biodegradable and benign solvents, others demonstrate the opposite (Hayyan et al., 2015; Hebert et al., 2016). In general terms, it has been assumed that they are not toxic and environmentally friendly because most of the DES components are naturally occurring, bio-renewable, biodegradable or can be bio-assimilated.

The standard protocols to study the cytotoxicity of new chemical components (such as the disk test) are not accurate in the case of DESs. The high viscosity, among other characteristics, makes the use of new methodologies to assess their biological toxicity essential. This microbiological study presents, for the first time, the continuous monitoring of various parameters (such as growth and pH) in liquid cultures with different concentrations of DES. This new protocol has revealed advantages in terms of biocompatibility, toxicity and tolerance.

2. Materials and Methods.

2.1. Synthesis of DES.

A recently described DES has been used in this study to optimize new protocols and guidelines for checking its toxicity. In brief, DES was synthesized using acetylcholine chloride (AcChCl):acetamide (1:2) as components. This was used as reaction medium to produce sulphonamides using a copper-catalysed process starting from triarylbismuthines, $Na_2S_2O_5$ and nitro compounds (Marset et al., 2019).

2.2. Microbial strain, culture media and incubation conditions.

The microorganism used in this study to test the toxicity, tolerance and the potential assimilation of the DES described here was *Escherichia coli* BL21 (DE3)

(Novagen). This microbe is a good example of a bacterial model organism extensively used in molecular biology, microbiology and genetic engineering due to its short generation time and large relevance in applied microbiology and biotechnology processes like protein overexpression (Kim et al., 2017).

The incubation experiments were conducted in 250 mL Erlenmeyers with 50 mL of Luria-Bertani medium (LB) following two strategies (Sezonov et al., 2007). On one hand, the initial pH of the culture media was adjusted to 7.0 using NaOH or HCl. Alternatively, the culture media were buffered with Tris-HCl 100 mM, adjusting the initial pH to 7.0. In all cases, pH was continuously monitored. The media was inoculated with 50 μL of a pre-inoculum of cells previously grown in standard LB media until the stationary phase of growth or with 50 μL of *E. coli* cells previously grown in LB media supplemented with DES at a lower concentration than the one tested in each assay. In all cases, the cells were grown at 37 °C with constant shaking (180 rpm). The growth of the bacterium was monitored by measuring the absorbance at 600 nm. All the physiological studies were done in triplicate.

In order to test the toxicity/tolerance of the DES, the media were supplemented with it, having final concentrations between 0 (control) and 750 mM. Likewise, the potential toxicity of the components that form this DES was also evaluated separately: acetylcholine chloride (AcChCl) and acetamide ($MeCONH_2$). The concentrations used were 600 mM and 1.2 M, respectively, which correspond to those contained in the mixture of DES at 600 mM (maximum concentration at which cell growth was detected). DES, acetylcholine chloride and acetamide were sterilised by following two approaches: they were either added to the culture media before autoclaving them at 120 °C for 30 minutes; or the DES was sterilized by UV and acetylcholine chloride and acetamide were filtered through 0.22 μm size filter and then added to the sterilised culture media.

The inhibition effect of the DES, AcChCl and $MeCONH_2$ was estimated by using the inhibition index (I) defined as: $I = (A_o - A) / A_o * 100\%$ (Kommanee et al., 2012), where A_o and A are the absorbances at 600 nm for the cultures in the absence and presence of the DES, AcChCl and $MeCONH_2$, respectively. Experiments were done by triplicate.

3. Results.

3.1. Analysis of pH changes in the culture media during the cell growth in the presence of DES.

A few studies about the toxicity of DES using bacteria as model organisms suggest that pH decreases significantly in culture media supplemented with DES, however, pH has not been continuously monitored in these studies (Dai et al., 2014). For this reason, the first analysis introduced in this study was the continuous checking of pH in buffered and unbuffered culture media supplemented with DES, acetylcholine chloride (AcChCl) or acetamide ($MeCONH_2$). The most dramatic pH depletion was detected in those unbuffered media in which DES or AcChCl were added to the culture media before autoclaving. Table 1 summarizes pH values at different time during the cell growth comparing buffered and unbuffered media. Even when the cultures were buffered, pH of the media containing DES or AcChCl decreased significantly (down to 4.4 in unbuffered DES-containing cultures and 4.5 in unbuffered and buffered AcChCl-containing cultures). The inoculation of the cultures and subsequent growth of the cells slightly increases the pH value as expected in the case of the controls and in the media containing $MeCONH_2$ (buffered and unbuffered).

[TABLE 1]

When the DES, AcChCl or $MeCONH_2$ were previously sterilised by UV or filtration respectively, and then added to the sterilised media, the changes of pH values were smaller (down to 5.3 as final pH in some of the cultures). In order to identify the magnitude of the contribution of autoclave and microbial metabolism to pH depletion, pH values were monitored in non-inoculated culture media containing two different concentrations of DES (150 mM and 600 mM). These cultures were incubated for the same period, under the same shaking and temperature conditions. After 24 hours of incubation, the final pH measured in sterile cultures with 600 mM of DES was 6.05 (Supplementary Table S1), which was much higher than the value for the same DES concentration inoculated with cells (Table 1).

From the results displayed in Table 1 and Supplementary Table S1, it is possible to conclude that the sterilization process based on autoclave may promote DES hydrolysis and probably reactions involving AcChCl, which contribute to pH acidification. DES hydrolysis and the consequent pH depletion also take place during the incubation, due to the culture media (Supplementary Table S1). *E. coli* growth and its metabolic activity is also involved in pH depletion.

3.2. Analysis of *E. coli* growth in the presence of different DES concentrations

To analyse *E. coli* tolerance and therefore the potential DES toxicity, cells were grown in buffered LB media supplemented with 600 mM DES and inoculated with *E. coli* cells grown in standard LB media (non-preadapted cells). This DES concentration was chosen based on the concentrations previously reported in the literature (Coleman et al., 2010). Cells were not able to grow under these conditions and the preliminary conclusion pointed out by the authors in this work was that 600 mM DES is toxic for this bacterium. It is important to highlight that microorganisms in general can induce specific reactions or pathways when they are exposed to compounds that are unusual in their natural environments or microcosmos in the laboratory. Considering this, several physiological studies were done to analyse the growth of pre-adapted *E. coli* cells. Thus, cells grown in the presence of 150 mM DES were used as inoculum for cultures containing 300 mM, etc. Figure 1 displays the growth curves of *E. coli* pre-adapted cells in LB containing different concentrations of DES.

[FIGURE 1]

The curves summarized in Figure 1 show two different patterns. The control curve shows the expected pattern in which a short Lag phase is followed by an exponential phase of growth (Lag phase) and then by a stationary phase of growth in which the maximum optical density (OD) observed was around 4. Shortly after 15 hours of incubation, the death phase starts. Curves from culture media containing 150 mM and 300 mM DES showed similar pattern and the final OD was close to 4. Regarding the cultures supplemented with 150 mM DES, two different curves can be

distinguished in Figure 1: one was obtained from culture media inoculated with cells from the standard culture (LB with no DES), and the other was measured from cultures in which cells used as inoculum were previously grown at 150 mM DES (preadapted cells). The differences found were clear; the culture inoculated with preadapted cells reduced its adaptation time (Lag phase) by two hours and cell duplication rate was increased (the slope of exponential phase of growth goes from 0.464 to 0.504). Therefore, better results in terms of cellular growth were obtained with preadapted cells thus suggesting that *E. coli* can deal with DES at least at concentrations between 0 and 300 mM. The cell inhibition rate remains below 6% in all these cases (Table 2).

In order to identify the maximum concentration of DES tolerated/assimilated by *E. coli*, the rest of the cultures with higher DES concentrations were always inoculated with cells pre-adapted to the concentration of DES immediately prior to the exposure. When the limit of 300 mM was exceeded, cellular adaptation started. At concentrations of 450 and 600 mM, the growth curves showed diauxic form and higher Lag phase. Diauxic curves have been previously reported from *E. coli* and many other bacteria (diphasic growth represented by two growth curves intervened by a short lag phase). These could be the result of: i) cellular growth usually using two different substrates at a different time (one of the substrates could be used preferentially and when it is exhausted, the second substrate is metabolised), or, ii) cellular growth affected by physicochemical parameters that can be changing during the incubation period. The maximum OD shown by the cultures with 450 mM DES was slightly higher (up to 4.5) than those reported from cultures with lower DES concentration. This profile could suggest that pre-adapted *E. coli* cells were able to grow nicely in the presence of 450 mM DES and may metabolise the DES or its derivatives when the DES is hydrolysed.

In the case of 600 mM, the percentage of cellular inhibition exceeded 30%, the Lag phase of growth increased significantly, and the final OD is half of the OD density reached by the other cultures. These results suggested that concentrations around 600 mM were toxic for the cells thus negatively affecting cellular growth and metabolic activities.

[TABLE 2]

The highest concentration tested in our experiments was 750 mM, at this concentration cell growth was not detected. Consequently, the DES concentrations above this value are toxic for *E. coli.*

3.3. Analysis of pH changes in culture media in the presence of the individual components of the DES: AcChCl and $MeCONH_2$ during *E. coli* growth

Regarding to the potential toxicity of the individual components of the DES here described (acetylcholine chloride – AcChCl – and acetamide – $MeCONH_2$ -), *E. coli* cells were grown in the presence of 600 mM DES (higher DES concentration allowing cellular growth) as well as in the presence of the two individual components at their final concentrations (600 mM AcChCl and 1.2 M $MeCONH_2$).

[FIGURE 2]

The value of the Lag phase is closed in the three cultures (DES, AcChCl and $MeCONH_2$; between 25 and 28 hours) but is higher than the Lag phase observed in control cultures. Final maximum OD in $MeCONH_2$-culture is higher than in control cultures. However, final OD in DES and AcChCl cultures (around 2.5) is lower than in control cultures. DES is more toxic than its constituent elements separately, based on the higher inhibition percentage and the lower duplication tax (Supplementary Table S2). pH decrease was observed in both AcChCl-cultures and $MeCONH_2$-cultures with 6.5 vs 7.4 at the end of the stationary phase of growth respectively. This depletion was higher in the presence of AcChCl, which correlates to the lower cellular growth detected in these cultures compared to $MeCONH_2$-cultures. Comparing the cellular rates from the DES, AcChCl and $MeCONH_2$ cultures, it can be concluded that the individual components are less toxic than the DES (mixture of two components in 1:2 ratio) as has been previously reported for most of DESs described up to now.

4. Discussion.

New advances on the knowledge of applied organic chemistry, biomedicine, pharmacy, nanotechnologies and materials sciences are contributing to the improvement of several industrial/technical processes at global scale. However, there is a concern about the importance of assessing risks of environmental pollutants obtained in those processes (Nilsen et al., 2019; Vita et al., 2018). Thus, several recent works address new guidelines and approaches to optimize the assessment of the quite recently environmental risks in both, terrestrial and aquatic environments (Song et al., 2018; Zhang et al., 2019; Shiyuan et al. 2019). This work contributes to the knowledge of assessing risks of environmental pollutants, as it is the case of DESs as potential environmental contaminants. The search of literature focused on DESs using PubMed or Scopus as search engines revealed that around 800 papers have been published during the last 15 years (date of access 18 July 2019). The first studies were reported at the beginning of this century, but 90% of them have been mainly published during the last 5 years. Considering the recent high impact of these kind of solvents, it is worthy to highlight that only around 50 of these studies, analyse or discuss about potential toxicity on DESs (PubMed; “Deep eutectic solvents” AND “toxicity” as keywords). The study of DES toxicity is a quite new topic for research. Thus, the first approach for testing toxicity of choline chloride (ChCl) based DESs used two Gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, and two Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. The cytotoxic effect of choline chloride (ChCl) based DESs was tested using the *Artemia salina* leach at that time (Hayyan et al., 2013a). Shortly after this study, some others stated that DESs toxicological effects are different based on their components, and usually, the cytotoxicity of tested DESs was much higher than that of their individual components (Hayyan et al., 2013b).

More recent studies have tested the toxicity of choline chloride-based DESs, with glucose, glycerol and oxalic acid as hydrogen bond donors, using fish and human cell lines, phytotoxicity using wheat and biodegradability using wastewater microorganisms through closed bottle test (Radošević et al., 2015). The cytotoxic potential of ammonium-based DESs with four hydrogen bond donors has also been studied using a variety of cell lines *in vitro* and animal model *in vivo* (Hayyan et al., 2015; Chen et al., 2017; Macário et al., 2019). Other organisms chosen as model organisms for testing DESs toxicity have been the following: *Allium sativum* (plant) and

hydra (invertebrate) (Wen et al., 2015), several eukaryotic microorganisms like *Phanerochaete chrysosporium*, *Aspergillus niger*, *Lentinus tigrinus* and *Candida cylindracea* (Juneidi et al., 2016) and fish cell lines (Radošević et al., 2016)

Although the examined DESs showed in general less cytotoxicity compared with ionic liquids (Kudłak et al., 2015), not all DESs can be considered readily biodegradable or environmentally friendly (Wen et al., 2015). Besides, most of the test used up to now to monitor DESs toxicity, are based on antibiograms and cells are not usually preadapted. Considering that DESs are normally highly dense and viscous solvents, the approaches already used in most of the cases do not guarantee accurate assays in which DESs could diffuse and interact efficiently with the cells or microbes. Consequently, more investigation on this matter are required in order to provide specific and optimised protocols for testing DESs toxicity thus avoiding misunderstanding or wrong conclusions about potential negative effects of DESs.

A deep analysis of all studies reported up to now about the toxicity and biodegradability of DESs reveals that:

- A large proportion of DESs are considered as “biodegradable” because most the components forming DESs are natural products. However, in most of the reports, accurate experiments to test biodegradability have not been conducted.

- The terms biodegradation, assimilation, toxicity, tolerance and recycling are usually used in a confusing way in several studies based on DESs. Biodegradation involves degradation, transformation or accumulation of DESs or its derivatives within the cells. Assimilation involves the use of DESs as a source of nutrients for the growth of cells. Tolerance implies an increase of the biomass in a specific range of concentrations, but above the maximum concentration, cell growth and its metabolism become inhibited.

- In many of the experiments done with microbial cells to test the toxicity of DESs, parameters like rate of growth, time of incubation, OD at stationary phase of growth, pH of the media in unbuffered and buffered media are not continuously monitored. This is also true of some studies previously done using bacteria as model organism (Wen et al., 2015). As reported here, the addition of DES to the culture media promotes pH decrease. In addition to this, the process to sterilise the culture media or DES stock is also directly connected to pH decrease. This phenomenon was

410 also previously described in the literature (Dai et al., 2014). In our study, pH depletion
411 due to DES hydrolysis or spontaneous reactions involving $MeCONH_2$, more significantly
412 affects cellular proliferation and metabolic rates, than the DES or its singular
413 components themselves.

414 - Toxicity can be measured by different approaches. In most of the reported
415 works, toxicity is checked by using solid culture media in plates with disks in which DES
416 is embedded (antibiogram-like test) (Zhao et al., 2015). This approach has an
417 important limitation due to the high density and viscosity characterising most of the
418 DESs described so far (Xu et al., 2017). These two properties restrict DESs diffusion
419 from the disk to its surroundings, thus giving results that may not reflect real
420 interaction between DESs and cells. Analysis of toxicity in liquid media minimizes this
421 negative impact, especially in those cases in which DESs are particularly dense or show
422 high viscosity.

423 - Biodegradability of DES (aerobic biodegradability) is usually evaluated using a
424 closed bottle test, in which DESs are added to aerobic aqueous media inoculated with
425 wastewater microorganisms, and the depletion of dissolved O_2 is periodically
426 determined (Coleman et al., 2010). The biodegradation test is normally considered as
427 valid if a 60% ThOD is achieved within 14 d for reference compound, and chemicals are
428 deemed to be readily biodegradable if they can reach a biodegradation level higher
429 than 60% within a 28-d period (OECD, 1992). However, most of the microbial strains
430 used in previous studies are microaerobic, anaerobic or can switch their metabolism
431 from highly oxic conditions to highly anoxic conditions. These aspects usually are not
432 considered in the test of biodegradability done with DESs reported so far.

433 - Crossed reactions between DESs and the salts/nutrients of the culture media
434 have not been considered in the toxicity studies. Hydrolysation of DESs could occur in
435 liquid culture media thus promoting crossing reactions between the products of the
436 DES hydrolysations and the salts, amino acids, carbohydrates, etc. within the culture
437 media. Besides, recent studies demonstrate that the DES water content, as well as the
438 formation of water shell layers are important in order to keep stable their
439 nanostructure (Hammond et al., 2017; Ma et al., 2018; Kumari et al., 2018).

440 - None of the studies reported up to now have considered testing the
441 preadaptation of cells to the DESs. Because of this, there is a loss of information in

terms of real capability of the cells to tolerate or even assimilate DESs. This study has demonstrated that under single assays in the presence of 600 mM DES the DES described here is toxic for the cells. However, when they were pre-adapted to this concentration, growth could be monitored. Consequently, we suggest including this parameter (cellular pre-adaptation) in further studies, in order to described more accurately the extent to which DESs are toxic to microbes.

5. Conclusions.

The new DES here tested is not toxic for *E. coli*, and maybe for other similar species, at concentrations between 0 and 300 mM. The DES used in this range of concentrations could be labelled as "environmentally friendly". At concentrations between 300 mM and 450 mM, cells can tolerate the DES, even though cellular growth and metabolic activities are slightly affected by it (typical microbial growth curves change to diauxic –diphasic- or triauxic –triphasic- curves and Lag times are higher than those observe at lower DES concentrations). Above 600 mM, the DES is toxic causing complete inhibition of growth. This toxicity is not only due to the chemical composition of the DES, but also due to the high acidification of the media caused by DES hydrolysis. Thus, concentrations above 600 mM could allow its use as bactericide or even to explore potential inhibitory effect on tumoral cells. The assays described here have demonstrated that the type of culture used to test toxicity, preadaptation of the cells used as a model to check cytotoxicity or the methods used to sterilise DESs can significantly affect the results when evaluating the degree of toxicity of DESs. Therefore, new guidelines and recommendations must be implemented in order to get accurate results when describing DES toxicity: i) liquid culture media vs solid media or antibiograms; ii) preadapted cells/tissues/seeds vs. non preadapted cells/tissues/seeds; iii) continuous monitoring of OD, pH, temperature, radiation, etc. during the cellular incubation in the presence of DES vs. the absence of continuous monitoring of these parameters, iv) buffered culture media vs. unbuffered cultures and v) sterilization of DES by UV or filtration (0.22 um filters) vs. sterilization by autoclave.

More investigation is still required, and some interesting questions arise for the next future: Which are the molecular bases beyond DESs toxicity? Are there extremophilic organisms able to deal with high concentrations of DESs?

References

Abbott, A.P., Boothby, D., Capper, G., Davies, D.L., Rasheed, R.K. 2004. Deep eutectic solvents formed between choline chloride and carboxylic acids: versatile alternatives to ionic liquids. J. Am. Chem. Soc. 126(29), 9142-9147. doi:10.1021/ja048266j

Abbott, A.P., El Ttaib, K., Frisch, G., McKenzie, K.J., Ryder, K.S. 2009. Electrodeposition of copper composites from deep eutectic solvents based on choline chloride. Phys. Chem. Chem. Phys. 11(21), 4269-4277. doi: 0.1039/b817881j

Alomar, M.K., Alsaadi, M.A., Jassam, T.M., Akib, S., Ali Hashim, M. 2017. Novel deep eutectic solvent-functionalized carbon nanotubes adsorbent for mercury removal from water. J. Colloid. Interface. Sci. 497, 413-421. doi:10.1016/j.jcis.2017.03.014

Aydin, F., Yilmaz, E., Soylak, M. 2018. Vortex assisted deep eutectic solvent (DES)-emulsification liquid-liquid microextraction of trace curcumin in food and herbal tea samples. Food. Chem. 243, 442-447. doi: 10.1016/j.foodchem.2017.09.154

Chen, J., Wang, Q., Liu, M., Zhang, L. 2017. The effect of deep eutectic solvent on the pharmacokinetics of salvianolic acid B in rats and its acute toxicity test. J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci. 1063, 60-66. doi: 10.1016/j.jchromb.2017.08.016

Chen, J., Wang, Y., Wei, X., Xu, P., Xu, W., Ni, R., Meng, J. 2018. Magnetic solid-phase extraction for the removal of mercury from water with ternary hydrosulphonyl-based deep eutectic solvent modified magnetic graphene oxide. Talanta. 188, 454-462. doi: 10.1016/j.talanta.2018.06.016

Coleman, D., Gathergood, N. 2010. Biodegradation studies of ionic liquids. Chem. Soc. Rev. 39(2), 600-637. doi:10.1039/b817717c

Cvjetko Bubalo, M., Ćurko, N., Tomašević, M., Kovačević Ganić, K., Radojčić Redovniković, I. 2016. Green extraction of grape skin phenolics by using deep eutectic solvents. Food. Chem. 200, 159-166. doi: 10.1016/j.foodchem.2016.01.040

Dai, Y., van Spronsen, J., Witkamp, G.J., Verpoorte, R., Choi, Y.H. 2013. Natural deep eutectic solvents as new potential media for green technology. Anal. Chim. Acta. 766, 61-68. doi:10.1016/j.aca.2012.12.013

Dai, Y., Verpoorte, R., Choi, Y.H. 2014. Natural deep eutectic solvents providing enhanced stability of natural colorants from safflower (*Carthamus tinctorius*). Food. Chem. 159, 116-121. doi:10.1016/j.foodchem.2014.02.155

García, A., Rodríguez-Juan, E., Rodríguez-Gutiérrez, G., Ríos, J.J., Fernández-Bolaños, J. 2016. Extraction of phenolic compounds from virgin olive oil by deep eutectic solvents (DESs). Food. Chem. 197(Pt A), 554-561. doi:10.1016/j.foodchem.2015.10.131

Gorke, J.T., Srienc, F., Kazlauskas, R.J. 2008. Hydrolase-catalyzed biotransformations in deep eutectic solvents. Chem. Commun (Camb). 10, 1235-1237. doi: 10.1039/b716317g

Gotor-Fernández, V., Paul, C.E. 2019. Deep eutectic solvents for redox biocatalysis. J. Biotechnol. 293, 24-35. doi:10.1016/j.jbiotec.2018.12.018

Gu, T., Zhang, M., Tan, T., Chen, J., Li, Z., Zhang, Q., Qiu, H. 2014. Deep eutectic solvents as novel extraction media for phenolic compounds from model oil. Chem. Commun (Camb). 50(79), 11746-11752. doi:10.1039/c4cc04661g

Gutiérrez, A., Aparicio, S., Atilhan, M. 2019. Design of arginine-based therapeutic deep eutectic solvents as drug solubilization vehicles for active pharmaceutical ingredients. Phys. Chem. Chem. Phys. 21(20), 10621-10634. doi:10.1039/c9cp01408j

Gutiérrez, M.C., Ferrer, M.L., Yuste, L., Rojo, F., del Monte, F. 2010. Bacteria incorporation in deep-eutectic solvents through freeze-drying. Angew. Chem. Int. Ed. Engl. 49(12), 2158-2162. doi:10.1002/anie.200905212

Hammond, O.S., Bowron, D.T., Edler, K.J. 2017. The Effect of Water upon Deep Eutectic Solvent Nanostructure: An Unusual Transition from Ionic Mixture to Aqueous Solution. Angew Chem Int. Ed. Engl. 56(33), 9782-9785. doi: 10.1002/anie.201702486.

Hayyan, M., Hashim, M.A., Hayyan, A., Al-Saadi, M.A., AlNashef, I.M., Mirghani, M.E., Saheed, O.K. 2013a. Are deep eutectic solvents benign or toxic? Chemosphere. 90(7), 2193-2195. doi:10.1016/j.chemosphere.2012.11.004

Hayyan, M., Hashim, M.A., Al-Saadi, M.A., Hayyan, A., AlNashef, I.M., Mirghani, M.E. 2013b. Assessment of cytotoxicity and toxicity for phosphonium-based deep eutectic solvents. Chemosphere. 93(2), 455-459. doi: 10.1016/j.chemosphere.2013.05.013

Hayyan, M., Looi, C. Y., Hayyan, A., Wong, W. F. & Hashim, M. A. 2015. *In Vitro* and *In Vivo* toxicity profiling of ammonium-based deep eutectic solvents. PLoS One 10(2), e0117934/0117931-e0117934/0117918, doi:10.1371/journal.pone.0117934

Hébert, M., Petiot, P., Benoit, E., Dansereau, J., Ahmad, T., Le Roch, A., Ottenwaelder, X., Gagnon, A. 2016. Synthesis of highly functionalized triarylbismuthines by functional group manipulation and use in palladium- and copper-catalyzed arylation reactions. J. Org. Chem. 81(13), 5401-5416. doi:10.1021/acs.joc.6b00767

Huang, J., Guo, X., Xu, T., Fan, L., Zhou, X., Wu, S. 2019. Ionic deep eutectic solvents for the extraction and separation of natural products. J. Chromatogr. A. 1598, 1-19. doi:10.1016/j.chroma.2019.03.046

Jablonský, M., Škulcová, A., Malvis, A., Šima, J. 2018. Extraction of value-added components from food industry based and agro-forest biowastes by deep eutectic solvents. J. Biotechnol. 282, 46-66. doi: 10.1016/j.jbiotec.2018.06.349

Juneidi, I., Hayyan, M., Mohd, Ali O. 2016. Toxicity profile of choline chloride-based deep eutectic solvents for fungi and *Cyprinus* carpio fish. Environ. Sci. Pollut. Res. Int. 23(8), 7648-7659. doi: 10.1007/s11356-015-6003-4

Kim, S., Jeong, H., Kim, E.Y., Kim, J.F., Lee, S.Y., Yoon, S.H. 2017. Genomic and transcriptomic landscape of *Escherichia coli* BL21(DE3). Nucleic Acids. Res. 45(9), 5285-5293. doi:10.1093/nar/gkx228

Kommanee, J., Preecharram, S., Daduang, S., Temsiripong, Y., Dhiravisit, A., Yamada, Y., Thammasirirak, S. 2012. Antibacterial activity of plasma from crocodile (*Crocodylus siamensis*) against pathogenic bacteria. Ann. Clin. Microbiol. Antimicrob. 11:22. doi:10.1186/1476-0711-11-22

Kudłak, B., Owczarek, K., Namieśnik, J. 2015. Selected issues related to the toxicity of ionic liquids and deep eutectic solvents-a review. Environ. Sci. Pollut. Res. Int. 22(16), 11975-11992. doi: 10.1007/s11356-015-4794-y

Kumari, P., Shobhna, Kaur, S, Kashyap, H.K. 2018. Influence of Hydration on the Structure of Reline Deep Eutectic Solvent: A Molecular Dynamics Study. ACS Omega. 3(11), 15246-15255. doi: 10.1021/acsomega.8b02447.

Li, X., Row, K.H. 2016. Development of deep eutectic solvents applied in extraction and separation. J. Sep. Sci. 39(18), 3505-3520. doi: 10.1002/jssc.201600633

Lindberg, D., de la Fuente Revenga, M., Widersten, M. 2010. Deep eutectic solvents (DESs) are viable cosolvents for enzyme-catalyzed epoxide hydorlysis. J. Biotechnol. 147(3-4), 169-171. doi:10.1016/j.jbiotec.2010.04.011

Lynam, J.G., Kumar, N., Wong, M.J. 2017. Deep eutectic solvents' ability to solubilize lignin, cellulose, and hemicellulose; thermal stability; and density. Bioresour. Technol. 238, 684-689. doi:10.1016.j.biortech.2017.04.079

Ma, C., Laaksonen, A. , Liu, C., Lu, X. , Ji, X. 2018. The peculiar effect of water on ionic liquids and deep eutectic solvents. Chem. Soc. Rev. 47(23), 8685-8720. doi: 10.1039/c8cs00325d.

Macário, I.P.E., Oliveira, H., Menezes, A.C., Ventura, S.P.M., Pereira, J.L., Gonçalves, A.M.M., Coutinho, J.A.P., Gonçalves, F.J.M. 2019. Cytotoxicity profiling of deep eutectic solvents to human skin cells. Sci Rep. 9(1):3932. doi: 10.1038/s41598-019-39910-y

Mamajanov, I., Engelhart, A.E., Bean, H.D., Hud, N.V. 2010. DNA and RNA in anhydrous media: duplex, triplex, and G-quadruplex secondary structures in a deep eutectic solvent. Angew. Chem. Int. Ed. Engl. 49(36), 6310-6314. doi:10.1002/anie.201001561

Marset, X., Torregrosa-Crespo, J., Martínez-Espinosa, R.M., Guillena, G., Ramón, D.J. 2019. Multicomponent synthesis of sulfonamides from triarylbismuthines, nitro compounds and sodium metabisulfite in deep eutectic solvents. Green Chem. 21, 4127-4132. doi:10.1039/c9gc01541h

Mbous, Y.P., Hayyan, M., Hayyan, A., Wong, W.F., Hashim, M.A., Looi, C.Y. 2017. Applications of deep eutectic solvents in biotechnology and bioengineering-Promises and challenges. Biotechnol. Adv. 35(2), 105-134. doi: 10.1016/j.biotechadv.2016.11.006

630 Morrison, H.G., Sun, C.C., Neervannan, S. 2009. Characterization of thermal behavior
631 of deep eutectic solvents and their potential as drug solubilization vehicles. Int. J.
632 Pharm. 378(1-2), 136-139. doi:10.1016/j.ijpharm.2009.05.039
633
634 Nilsen, E., Smalling, K.L., Ahrens, L., Gros, M., Miglioranza, K.S.B., Picó, Y., Schoenfuss,
635 H.L. 2019. Critical review: Grand challenges in assessing the adverse effects of
636 contaminants of emerging concern on aquatic food webs. Environ. Toxicol. Chem.
637 38(1), 46-60. doi: 10.1002/etc.4290.
638
639 Nkuku, C.A., LeSuer, R.J. 2007. Electrochemistry in deep eutectic solvents. J. Phys.
640 Chem. B. 111(46), 13271-13277. doi:10.1021/jp075794j
641
642 OECD. 1992. Guideline for testing of chemicals, adopted by the Council on 17th July
643 1992 Ready Biodegradability (https://www.oecd.org/chemicalsafety/risk-
644 assessment/1948209.pdf)
645
646 Pätzold, M., Siebenhaller, S., Kara, S., Liese, A., Syldatck, C., Holtmann, D. 2019. Deep
647 eutectic solvents as efficient solvents in biocatalysis. Trends. Biotechol. pii:S0167-
648 7799(19)30052-6. doi:10.1016./j.tibtech.2019.03.007
649
650 Pedro, S.N., Freire, M.G., Freire, C.S.R., Silvestre, A.J.D. 2019. Deep eutectic solvents
651 comprising active pharmaceutical ingredients in the development of drug delivery
652 systems. Expert. Opin. Drug. Deliv. 16(5), 497-506.
653 doi:10.1080/17425247.2019.1604680.
654
655 Pena-Pereira, F., Namiesnik, J. 2014. Ionic liquids and deep eutectic mixtures:
656 sustainable solvents for extraction processes. Chem. Sus. Chem. 7(7), 1784-1800. doi:
657 10.1002/cssc.201301192
658
659 Procentese, A., Raganati, F., Olivieri, G., Russo, M.E., Rehmann, L., Marzocchella, A.
660 2018. Deep eutectic solvents pretreatment of agro-industrial food waste. Biotechnol.
661 Biofuels. 11:37. doi: 10.1186/s13068-018-1034-y

Radošević, K., Bubalo, M.C., Srček, V.G., Grgas, D., Dragičević, T.L., Redovniković, I.R. 2015. Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents. Ecotoxicol. Environ. Saf. 112, 46-53. doi:10.1016/j.ecoenv.2014.09.034

Radošević, K., Železnjak, J., Cvjetko Bubalo, M., Radojčić Redovniković, I., Slivac, I., Gaurina Srček V. 2016. Comparative *in vitro* study of cholinium-based ionic liquids and deep eutectic solvents toward fish cell line. Ecotoxicol. Environ. Saf. 131, 30-36. doi:10.1016/j.ecoenv.2016.05.005

Santos, F., P. S. Leitão, M.I, C. Duarte, A.R. 2018. Properties of therapeutic deep eutectic solvents of l-arginine and ethambutol for tuberculosis treatment. Molecules. 24(1), pii: E55. doi: 10.3390/molecules24010055

Saravana, P.S., Ho, T.C., Chae, S.J., Cho, Y.J., Park, J.S., Lee, H.J., Chun, B.S. 2018. Deep eutectic solvent-based extraction and fabrication of chitin films from crustacean waste. Carbohydr. Polym. 195, 622-630. doi:10.1016/j.carbpol.2018.05.018

Sezonov, G., Joseleau-Petit, D., D'Ari, R. 2007. *Escherichia coli* physiology in Luria-Bertani broth. J. Bacteriol. 189(23), 8746-8749. doi:10.1128/JB.01368-07

Sheng, Q., Liu, R., Zheng, J. 2012. Prussian blue nanospheres synthesized in deep eutectic solvents. Nanoscale. 4(21), 6880-6886. doi:10.1039/c2nr31830j.

Song, B., Xu, P., Zeng, G., Gong, J., Wang, X., Yan, J., Wang, S., Zhang, P., Cao, W., Ye, S. 2018. Modeling the transport of sodium dodecyl benzene sulfonate in riverine sediment in the presence of multi-walled carbon nanotubes. Water Res. 129, 20-28. doi: 10.1016/j.watres.2017.11.003.

Takla, S.S., Shawky, E., Hammoda, H.M., Darwish, F.A. 2018. Green techniques in comparison to conventional ones in the extraction of Amaryllidaceae alkaloids: best

694 solvents selection and parameters optimization. J. Chromatogr. A. 1567, 99-110.
695 doi:10.1016/j.chroma.2018.07.009
696
697 Tang, W., Gao, F., Duan, Y., Zhu, T., Ho Row, K. 2017. Exploration of deep eutectic
698 solvents-based molecularly imprinted polymers as solid-phase extraction sorbents for
699 screening chloramphenicol in milk. J. Chromatogr. Sci. 55(6), 654-661.
700 doi:10.1093/chromsci/bmx011
701
702 Vita, N.A., Brohem, C.A., Canavez, A.D.P.M., Oliveira, C.F.S., Kruger, O., Lorencini, M.,
703 Carvalho, C.M. 2018. Parameters for assessing the aquatic environmental impact of
704 cosmetic products. Toxicol Lett. 287, 70-82. doi: 10.1016/j.toxlet.2018.01.015.
705
706 Wagle, D.V., Zhao, H., Baker, G.A. 2014. Deep eutectic solvents: sustainable media for
707 nanoscale and fucntional materials. Acc. Chem. Res. 47(8), 2299-2308.
708 doi:10.1021/ar5000488
709
710 Wen, Q., Chen, J.X., Tang, Y.L., Wang, J., Yang, Z. 2015. Assessing the toxicity and
711 biodegradability of deep eutectic solvents. Chemosphere. 132, 63-69.
712 doi:10.1016/j.chemosphere.2015.02.061
713
714 Xu, P., Zheng, G.W., Zong, M.H., Li, N., Lou, W.Y. 2017. Recent progress on deep
715 eutectic solvents in biocatalysis. Bioresources and bioprocessing. 4:34.
716 doi:10.1186/s40643-017-0165-5
717
718 Yoo, D.E., Jeong, K.M., Han, S.Y., Kim, E.M., Jin, Y., Lee, J. 2018. Deep eutectic solvent-
719 based valorization of spent coffee grounds. Food. Chem. 255, 357-364. doi:
720 10.1016/j.foodchem.2018.02.096
721
722 Zdanowicz, M., Wilpiszewska, K., Spychaj, T. 2018. Deep eutectic solvents for
723 polysaccharides processing: a review. Carhoydr. Polym. 200, 361-380.
724 doi:10.1016/j.carbpol.2018.07.078
725

Zeng, Q., Wang, Y., Huang, Y., Ding, X., Chen, J., Xu, K. 2014. Deep eutectic solvents as novel extraction media for protein partitioning. Analyst. 139(10), 2565-2573. doi:10.1039/c3an02235h

Zhang, H., Wang, Y., Zhou, Y., Chen, J., Wei, X., Xu, P. 2018. Aqueous biphasic systems formed by deep eutectic solvent and new-type salts for the high-performance extraction of pigments. Talanta. 181, 210-216. doi:10.1016/j.talanta.2018.01.014

Zhang, M., Bradford, S.A., Šimůnek, J., Vereecken, H., Klumpp, E. 2019. Co-transport of multi-walled carbon nanotubes and sodium dodecylbenzenesulfonate in chemically heterogeneous porous media. Environ. Pollut. 247, 907-916. doi: 10.1016/j.envpol.2019.01.106.

Zhang, Q., De Oliveira Vigier, K., Royer, S., Jérôme, F. 2012. Deep eutectic solvents: syntheses, properties and applications. Chem. Soc. Rev. 41(21), 7108-7146. doi:10.1039/c2cs35178a

Zhao, H., Baker, G.A., Holmes, S. 2011. Protease activation in glycerol-based deep eutectic solvents. J. Mol. Catal. B. Enzym. 72(3-4), 163-167. doi:10.1016/j.molcatb.2011.05.015

Zhao, C., Qu, X. 2013a. Recent progress in G-quadruplex DNA in deep eutectic solvent. Methods. 64(1), 52-58. doi:10.1016/j.ymeth.2013.04.017

Zhao, C., Ren, J., Qu, X. 2013b. G-quadruplexes form ultrastable parallel structures in deep eutectic solvent. Langmuir. 29(4), 1183-1191. doi:10.1021/la3043186

Zhao, B.Y., Xu, P., Yang, F.X., Wu, H., Zong, M.H., Lou, W.Y. 2015. Biocompatible deep eutectic solvents based on choline chloride: characterization and application to the

extraction of rutin from *Sophora japonica*. ACS Sustainable Chem. Eng. 3(11), 2746-2755. doi:10.1021/acssuschemeng.5b00619

Zhou, S., Zheng, Z.; Mei, T.; Wang, X. 2019. Structural design and material preparation of carbon-based electrodes for high-performance lithium storage systems. Carbon, 144, 1-7.

Zhu, S., Liu, D., Zhu, X., Su, A., Zhang, H. 2017. Extraction of illegal dyes from red chili peppers with cholinium-based deep eutectic solvents. J. Anal. Methods. Chem. 2017, 2753752. doi: 10.1155/2017/2753752

Zhuang, B., Dou, L.L., Li, P., Liu, E.H. 2017. Deep eutectic solvents as green media for extraction of flavonoid glycosides and aglycones from *Platycladi Cacumen*. J. Pharm. Biomed. Anal. 134, 214-219. doi:10.1016/j.jpba.2016.11.049

Acknowledgements

This work was supported by the University of Alicante (VIGROB-173 and VIGROB-309), the Spanish Ministerio de Economía, Industria y Competitividad (CTQ2015-66624-P, PGC2018-096616-B-I00 and RTI2018-099860-B-I00). X.M. and J.T.C. thank Generalitat Valenciana (ACIF/2016/057 and ACIF/2016/077) for their fellowships. Thanks to T. Coyle for English checking of the manuscript.

Author contributions

R.M.M., D. J. R. and G. G. directed and supervised the project. X. M. designed and performed the synthetic experiments, characterized all products and performed the DES physicochemical characterization. J.T.C. and R.M.M. designed and performed the experiments related to the biological evaluation of the DES. All the authors prepared the manuscript and they have agreed to the content of it.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Table 1: Average of pH values from three biological replicates of buffered and unbuffered culture media during the cell growth. (*) means "buffered". AcChCl corresponds to acetylcholine chloride and $MeCONH_2$ to acetamide. Final DES concentration was 600 mM. When AcChCl and $MeCONH_2$ were tested separately, their final concentrations were those contained in the mixture of DES at 600 mM (see materials and methods). C corresponds to control media in which LB is not supplemented neither DES nor AcChCl or $MeCONH_2$. In all cases, DES, AcChCl and $MeCONH_2$ were added to the culture media before autoclaving.

Time (h of incubation)	**C**	**C***	**DES**	**DES***	**AcChCl**	**AcChCl***	**$MeCONH_2$**	**$MeCONH_2$***
0 before autoclave	7.0	7.0	6.9	6.9	6.9	6.9	6.9	7 .0
0 after autoclave and before cells inoculation	7.2	7.0	4.5	4.7	4.5	4.7	6.6	7.0
5.0	7.5	6.8	4.4	4.6	4.5	4.5	6.4	6.9
10.3	8.0	7.1	4.5	4.6	4.4	4.6	6.9	6.8
23.0	8.5	8.1	4.5	4.7	4.4	4.6	8.4	7.7
48.0	8.4	8.1	4.5	4.8	4.5	4.7	8.5	7.9

Table 2: Summary of parameters regarding cellular growth and percentage of growth inhibition in culture media supplemented with different concentrations of the DES. The DES was sterilised by UV and then added to sterilised cultures. * Indicates that cells used as inoculum were not pre-adapted.

[DES] (mM)	**SLOPE (ΔAbs/hour) (Rate of cellular duplication)**	**R^2**	**I (%) (Percentage of growth inhibition**	**Lag time (hours)**	**Average of pH values during incubation**	**Average of minimal pH value detected during incubation**
0	0.67	0.99	0	3.75	6.87	6.77

150*	0.46	0.99	4.76	7.50	6.65	6.61
150	0.50	0.97	5.57	5.50	6.53	6.53
300	0.40	0.99	0.00	2.00	6.72	6.63
450	0.43	0.97	0.00	28.50	6.60	6.10
600	0.53	0.94	32.57	28.50	5.80	5.80
750	0	0	98.89	-	-	5.77

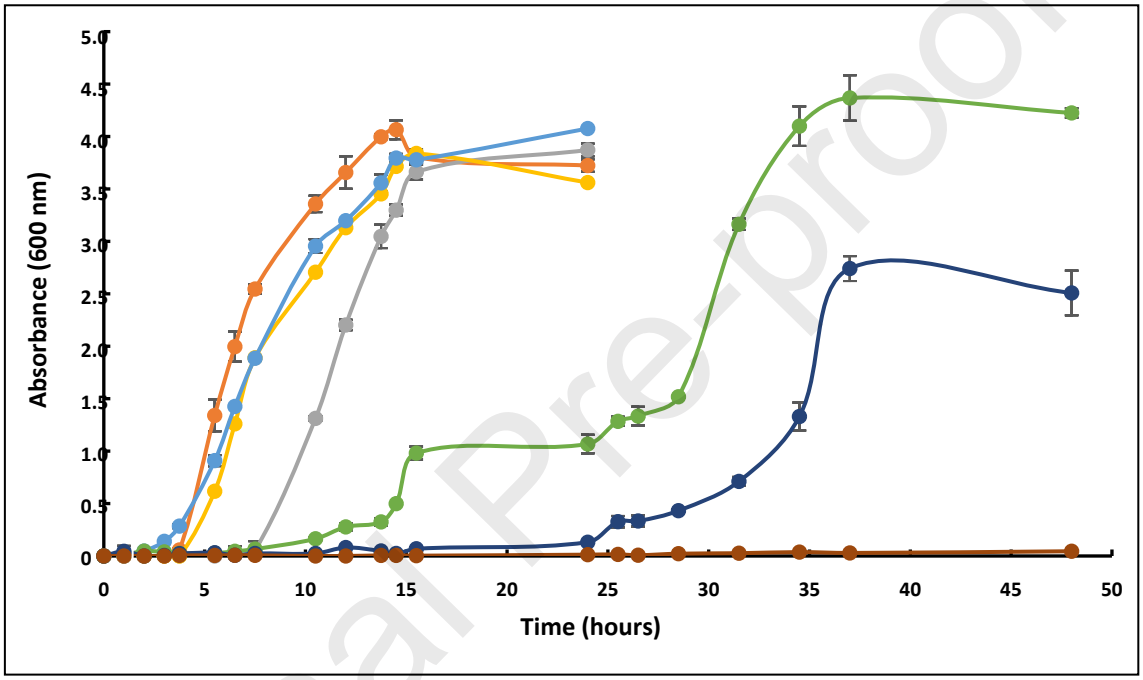

Figure 1: *E. coli* growth curves. Cells were grown in LB media supplemented with different concentrations of DES. Legend of colours: Orange = control (LB media without DES); Yellow = LB + 150 mM DES inoculated with pre-adapted cells; Grey = LB + 150 mM DES inoculated with non-pre-adapted cells; light blue = LB + 300 mM DES and preadapted cells; green = LB + 450 mM DES and preadapted cells; dark blue = LB + 600 mM DES and preadapted cells; dark red = LB + 750 mM DES and preadapted cells. For each curve, the dots correspond to the average of three values obtained from three biological replicates, standard deviation of these values was between ± 0.01 and ± 0.16.

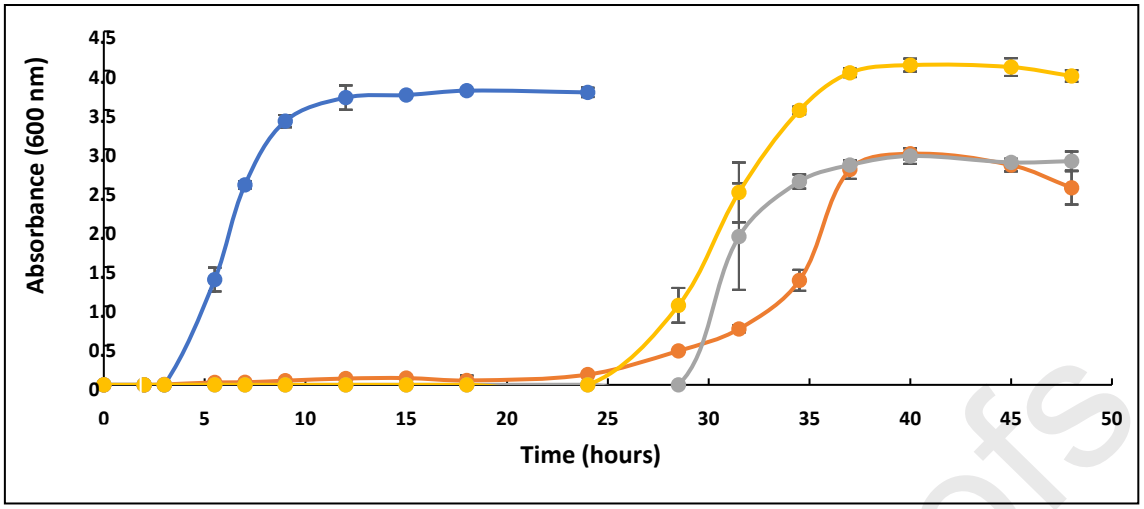

Figure 2: *E. coli* growth curves. Cells were grown in buffered LB media supplemented with 600 mM DES, 600 mM AcChCl or 1.2 M $MeCONH_2$. DES was sterilised by UV and then added to sterilised media. AcChCl and $MeCONH_2$ were sterilised by filtration and then added to sterilised media. Legend of colours: Blue = control (LB media without DES); Purple = LB + 1.2 $MeCONH_2$; Green = LB + 600 mM AcChCl; Red = LB + 600 mM DES. In all cases, cultures were inoculated with pre-adapted cells. For each curve, the dots correspond to the average of three values obtained from three biological replicates, standard deviations were between ± 0.01 and ± 0.2.

SUPPLEMENTARY MATERIALS:

Supplementary Table 1: pH values in buffered media supplemented with 150 mM or 600 mM DES. DES was sterilised by UV and then added to sterilised cultures. These cultures were not inoculated with cells. SD values were ± 0.1 in all cases.

Time (hours of incubation)	150 mM DES	600 mM DES
0	7.00	7.00
2	6.80	6.90
6	6.65	6.75
10	6.52	6.35
15	6.41	6.25
24	6.27	6.05
48	5.65	5.30

Supplementary Table 2: summary of parameters related to cellular growth and percentage of growth inhibition in culture media supplemented with DES (600 Mm), acetylcholine chloride (AC) and acetamide (AT). DES was sterilized by UV while AC and AT were filtered using a 0,22-micron filter and then added to sterilized culture media.

Culture	SLOPE (ΔAbs/hour)	R^2	I (%)	Lag time	Average of	Average of

	(Rate of cellular duplication)		(Percentage of growth inhibition	(hours)	pH values during incubation	minimal pH value detected during incubation
Control	0.58	0.99	0.00	5.50	6.77	6.77
DES (600 mM)	0.36	0.93	21.44	5.50	6.34	5.87
AC	0.43	0.93	22.21	28.50	7.15	5.81
AT	0.34	0.98	0.00	28.50	6.10	6.10

881

882

883

884 **Highlights**

885

886 • DESs are promising compounds for organic chemistry and pharmacy.

887 • Despite their advantages as reaction media, there is controversy about their toxicity.

888 • Their toxicity was mainly assayed by antibiograms, which is not an accurate approach
889 for DESs.

890 • New guidelines for DESs toxicity monitoring are presented.

891 • Protocols to test DES toxicity may lead to errors in assigning their eco-friendly nature.

892

893

894

895

896 **Conflict of interest**

897 The authors declare that the research was conducted in the absence of any
898 commercial or financial relationships that could be construed as a potential conflict of
899 interest.

900

901